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Incidence of *Klebsiella* Species in Surface Waters and Their Expression of Virulence Factors

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To investigate the occurrence of different *Klebsiella* spp. in aquatic environments, a total of 208 samples of natural surface waters was examined. From half (53%) of these samples, 123 *Klebsiella* strains were isolated, the most common species being *Klebsiella pneumoniae*. A comparison of these isolates to a group of 207 clinical *K. pneumoniae* isolates demonstrated that water isolates of *K. pneumoniae*, unlike those of *K. oxytoca* and *K. planticola*, are as capable as clinical isolates of expressing putative virulence factors such as serum resistance and capsular polysaccharides, pili, and siderophores.

Bacteria of the genus *Klebsiella* are a frequent cause of nosocomial infections (8). *Klebsiella* spp. are ubiquitous in nature. Their nonclinical habitats encompass the gastrointestinal tract of mammals as well as environmental sources such as soil, surface waters, and plants (1). Environmental isolates have been described as being indistinguishable from human clinical isolates with respect to their biochemical reactions and virulence (12). While the medical significance of *Klebsiella* obtained in the natural environment is far from clear, such habitats are thought to be potential reservoirs for the growth and spread of these bacteria which may colonize animals and humans (11).

At present the genus *Klebsiella* is subdivided into five species. Clinically, the most important species are *Klebsiella pneumoniae* and *K. oxytoca*, while *K. ornithinolytica, K. terrigena*, and *K. planticola* are rarely isolated from human clinical specimens (6, 18). *K. planticola* and *K. terrigena* are considered to be environmental species, as reflected in their species designations. In contrast to *K. pneumoniae*, neither species grows at elevated temperatures, such as at 44.5°C.

To date, however, studies on the frequency of *Klebsiella* in nonclinical habitats have focused on *K. pneumoniae* or have not identified isolates to the species level at all (2, 3, 4, 5, 10, 11, 12, 19). No data are currently available on the incidence of the various *Klebsiella* spp. in environmental habitats.

The purpose of the present study was to determine the occurrence and distribution of the five *Klebsiella* species in natural surface waters. Having done this, we investigated whether environmental isolates are capable of producing putative *Klebsiella* virulence factors, such as pili, serum resistance properties, siderophores, or particular capsular types. For comparison, a group of previously described *K. pneumoniae* human clinical isolates was used (15).

Collection of water samples. From November 1997 to June 1998, 208 water samples were collected in sterile 250-ml glass bottles from 196 different sampling sites at streams, lakes, and the Baltic Sea in various geographic areas of Schleswig-Holstein, Germany. Samples were taken 30 cm below the water surface, stored on ice for transportation, and processed for bacteriological analysis within 4 h of collection. The samples were classified as freshwater (conductivity, <1,500 μS/cm), brackish water (1,500 to 15,000 μS/cm), or salt water (>15,000 μS/cm).

Isolation of strains. Each 250-ml sample was filtered through a 0.45-μm-pore-size membrane (Sartorius, Göttingen, Germany). The membranes were transferred onto Simmons citrate agar with 1% (wt/vol) inositol and incubated for 48 h at 37°C. This medium is highly selective but not inhibitory for the recovery of *Klebsiella* (21). Presumptive *Klebsiella* colonies were isolated, followed by identification according to the biochemical tests given by Ørskov (13), which include fermentation of melezitose and L-sorbose, gas production from lactose at 44.5°C, growth at 10°C, pectate degradation, and utilization of *m*-hydroxybenzoate and hydroxy-L-proline. A group of 207 human clinical *K. pneumoniae* isolates previously obtained from human infections was used for comparison (15).

Capsule typing. The isolates were serotyped by the capsular swelling method as described by Ullmann (20). Polyvalent rabbit anticapsule sera were used for screening, and monospecific sera were used for typing.

Hemagglutination assay. Expression of type 1 pili (mannose-sensitive hemagglutination [MSHA]) and type 3 pili (mannose-resistant *Klebsiella*-like hemagglutination [MR/K-HA]) was examined as described previously (16), with MSHA being assessed on guinea pig erythrocytes and MR/K-HA on tanned ox red blood cells. Bacteria were grown statically at 48-h intervals. Fifty microliters of bacterial suspensions (approximately 10^{11} bacteria/ml) and 50 μl of erythrocytes (5×10^8/ml) were mixed in porcelain tiles, rocked, and observed for 3 min at room temperature. Agglutination was finally read after further incubation for 10 min at 4°C.

Determination of siderophore production. For detection of enterobactin and aerobactin production the cross-feeding bioassay of Hantke (7) was performed as described elsewhere (14). Nutrient agar supplemented with 2,2′-dipyridyl (200 μM) served as iron-restricted agar medium. *Escherichia coli* strain H1887 (ColV$^-$ Aer$^-$ Iut$^+$ FepA$^-$ Fiu$^-$ Cir$^-$ *aroB*) was used as

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TABLE 1. Incidence of *Klebsiella* spp. in samples of different types of surface water

Organism	No. (%) of organisms in:		
	Freshwater (n = 133)	Brackish water (n = 12)	Salt water (n = 63)
K. pneumoniae	42 (31.6)	3 (25)[a]	17 (25.4)[a]
K. oxytoca	18 (12.8)[a]	2 (16.7)	14 (22.2)
K. planticola	17 (12.8)	1 (8.3)	9 (14.3)
Total	77 (51.9)[a]	6 (41.7)[a]	40 (57.1)[a]

[a] Detection of two different species or two different isolates of the same species in a single water sample.

TABLE 2. Distribution of capsule types in *Klebsiella* spp. from natural surface waters and from clinical specimens

K type	No. (%) of isolates with each K type			
	K. pneumoniae (n = 62)	*K. oxytoca* (n = 34)	*K. planticola* (n = 27)	Clinical *K. pneumoniae*[a] (n = 207)
K33	6 (9.7)	5 (14.7)	2 (7.4)	3 (1.4)
K69	8 (12.9)	2 (5.9)	0 (0)	1 (0.5)
K2	0 (0)	2 (5.9)	3 (11.1)	28 (13.5)
K70	0 (0)	2 (5.9)	2 (7.4)	0 (0)
K56	1 (1.6)	0 (0)	2 (7.4)	1 (0.5)
K13	4 (6.5)	0 (0)	0 (0)	2 (1.0)
K35	4 (6.5)	2 (5.9)	1 (3.7)	3 (1.4)
K23	1 (1.6)	2 (5.9)	0 (0)	2 (1.0)
K26	0 (0)	2 (5.9)	0 (0)	1 (0.5)
Other K types	<5% Each	<5% Each	<5% Each	<5% Each
Untypeable	6 (9.7)	1 (2.9)	0 (0)	16 (7.7)

[a] Comparison group of clinical *K. pneumoniae* isolates. Data are from reference 15.

the indicator strain for aerobactin production, and strain H1939 (FepA$^+$ Fiu$^-$ Cir$^-$ FhuA$^-$ FhuB$^-$ *aroB*) was used to indicate enterobactin production. Aerobactin production was counterchecked using the *E. coli* strain H1886, which is the Iut$^-$ parent strain of H1887. Each isolate was tested twice. The indicator strains were kindly provided by K. Hantke, University of Tübingen, Tübingen, Germany.

Serum bactericidal assay. The susceptibility of bacteria to human serum was determined by the method of Hughes et al. (9) as slightly modified previously (17). Twenty-five microliters of bacterial suspensions (2×10^6 cells/ml) and 75 μl of normal human serum were put into microtiter trays and incubated at 37°C. Viability was determined immediately and after 1, 2, and 3 h of incubation by plating on brain heart infusion agar for colony counts. Responses were graded as highly sensitive, intermediately sensitive, or serum resistant according to the system of Hughes and colleagues (9). Each strain was tested three times.

Statistical analysis. The significance of differences between groups of bacteria was evaluated by Yates' corrected chi square test for 2 by 2 contingency tables. Medians were compared using the nonparametric analysis of variance test of Kruskal-Wallis and by the Mann-Whitney test. All tests were performed using GraphPad InStat, version 3.00 (GraphPad Software, San Diego, Calif.).

Over an 8-month period, 208 samples of natural surface water were taken from 196 different sampling sites. One hundred ten of the water samples (52.9%) were found to contain 123 *Klebsiella* strains. Thirteen water samples each contained two different *Klebsiella* species, and two other samples contained two different strains of the same species. In our experience, the frequency of *Klebsiella* isolation from surface water depends to a large extent on the volume investigated. In a preliminary experiment using 1-ml samples, we were not able to isolate this bacterium from any of the 47 water samples examined. We therefore decided to investigate 250-ml samples. Even then, the number of *Klebsiella* colonies per sample was low (usually 1 to 5 CFU/250 ml). The incidence of positive water samples was conspicuously independent of source and season. We found no significant differences in the frequency of isolation between freshwater, salt water, and brackish water (Table 1). Likewise, no seasonal effects could be observed (data not shown). *K. pneumoniae* was most common (n = 62; 52%), followed by *K. oxytoca* (n = 34; 27%) and *K. planticola* (n = 27; 22%). Neither *K. ornithinolytica* nor *K. terrigena* was detected. With respect to the latter species, this stands in striking contrast to the view that *K. terrigena* is an environmental species that can be isolated from surface waters (1). The lack of *K. terrigena* in our surface water samples, however, was not due to a possible inhibiting effect of the *Klebsiella*-selective agar used since in preliminary experiments we confirmed that all *Klebsiella* species can grow on this medium. It is conceivable, though, that there are geographic differences in the occurrence of *K. terrigena*.

Serotyping revealed K types K33 and K69 as the most common capsular types among *K. pneumoniae* and *K. oxytoca* isolates (13 to 15%) (Table 2). Both serotypes were rarely observed (<2%) in the group of 207 clinical *K. pneumoniae* isolates investigated for comparison purposes. In contrast, clinical *K. pneumoniae* strains predominantly expressed the K antigen K2 (14%), which is considered to be a main determinant of *Klebsiella* virulence (18). Surprisingly, this serotype was also the most common K type among *K. planticola* isolates (11%).

Clinical *K. pneumoniae* strains and isolates from surface water were very similar with respect to the incidence of type 1 and type 3 pili. Both groups were significantly more often fimbriated than *K. oxytoca* or *K. planticola* strains ($P < 0.005$) (Table 3). However, the statistical significance of the differences between groups in the production of type 3 pili was only at a P value of 0.07 in some cases. MSHA expression in *K. oxytoca* was very rare (15%).

The incidences of serum resistance properties differed considerably between the groups of environmental isolates (Table 3). About half (53%) of the *K. oxytoca* strains proved to be serum resistant, whereas only 11% of the *K. pneumoniae* and 4% of the *K. planticola* isolates were resistant ($P < 0.0001$). Clinical *K. pneumoniae* strains exhibited serum resistance properties twice as often (25%) as environmental *K. pneumoniae* strains ($P < 0.05$).

Siderophore production by the bacterial groups was very similar. Except for five strains, all isolates investigated were able to synthesize the catechol-type siderophore enterobactin (Table 3). In contrast, production of the hydroxamate-type siderophore aerobactin was lacking (environmental isolates) or very rare (clinical *K. pneumoniae* strains).

To determine whether environmental species differ with re-

TABLE 3. Distribution of fimbriae, serum resistance properties, and siderophores among *Klebsiella* spp. from natural surface waters and from clinical specimens

Virulence factor	No. (%) of positive isolates			
	K. pneumoniae (n = 62)	*K. oxytoca* (n = 34)	*K. planticola* (n = 27)	Clinical *K. pneumoniae*[a] (n = 207)
MSHA (type 1 fimbriae)	54 (87)	5 (15)	16 (59)	177 (86)
MR/K-HA (type 3 fimbriae)	45 (73)	18 (53)	8 (30)	145 (70)
Serum resistance	7 (11)	18 (53)	1 (3.7)	52 (25)
Production of:				
Enterobactin	60 (97)	33 (97)	27 (100)	205 (99)
Aerobactin	0 (0)	0 (0)	0 (0)	11 (5.3)
Mean cumulative no. of virulence factors per isolate	2.7	2.2	1.9	2.9

[a] Comparison group of clinical *K. pneumoniae* isolates. Data are from reference 15.

spect to the expression of virulence factors and whether they differ in this respect from clinical *K. pneumoniae* isolates, the factors detected in each strain (MSHA, MR/K-HA, serum resistance, enterobactin synthesis, and aerobactin production) were added up to get the cumulative number of virulence factors expressed per isolate (Table 3). The mean number of factors expressed by environmental *K. oxytoca* (2.2) and *K. planticola* (1.9) strains was significantly lower than that expressed by environmental (2.7) or clinical (2.9) *K. pneumoniae* isolates ($P < 0.01$).

To sum up, a high percentage (53%) of surface water samples proved to be positive for *Klebsiella* spp., the most common species being *K. pneumoniae*. Furthermore, our data show that surface water isolates of *K. pneumoniae* resemble clinical strains in the expression of virulence factors, whereas water isolates of *K. oxytoca* and *K. planticola* differ from clinical strains in this respect. With respect to the factors examined, we found no evidence that environmental *K. pneumoniae* strains are less virulent than clinical strains. Whether this finding has any relevance to public health is at present unclear and should be evaluated by further studies.

REFERENCES

1. **Bagley, S. T.** 1985. Habitat association of *Klebsiella* species. Infect. Control **6:**52–58.
2. **Bagley, S. T., R. J. Seidler, H. W. Talbot, Jr., and J. E. Morrow.** 1978. Isolation of *Klebsielleae* from within living wood. Appl. Environ. Microbiol. **36:**178–185.
3. **Brown, C., and R. J. Seidler.** 1973. Potential pathogens in the environment: *Klebsiella pneumoniae*, a taxonomic and ecological enigma. Appl. Microbiol. **25:**900–904.
4. **Campbell, L. M., G. Michaels, R. D. Klein, and I. L. Roth.** 1976. Isolation of *Klebsiella pneumoniae* from lake water. Can. J. Microbiol. **22:**1762–1767.
5. **Edberg, S. C., V. Piscitelli, and M. Cartter.** 1986. Phenotypic characteristics of coliform and noncoliform bacteria from a public water supply compared with regional and national clinical species. Appl. Environ. Microbiol. **52:**474–478.
6. **Farmer, J. J., III, B. R. Davis, F. W. Hickman-Brenner, A. McWhorter, G. P. Huntley-Carter, M. A. Asbury, C. Riddle, H. G. Wathen-Grady, C. Elias, G. R. Fanning, A. G. Steigerwalt, C. M. O'Hara, G. K. Morris, P. B. Smith, and D. J. Brenner.** 1985. Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens. J. Clin. Microbiol. **21:**46–76.
7. **Hantke, K.** 1990. Dihydroxybenzoylserine—a siderophore for *E. coli*. FEMS Microbiol. Lett. **55:**5–8.
8. **Horan, T., D. Culver, W. Jarvis, G. Emori, S. Banerjee, W. Martone, and C. Thornsberry.** 1988. Pathogens causing nosocomial infections. Antimicrob. Newsl. **5:**65–67.
9. **Hughes, C., R. Phillips, and A. P. Roberts.** 1982. Serum resistance among *Escherichia coli* strains causing urinary tract infection in relation to O type and the carriage of hemolysin, colicin, and antibiotic resistance determinants. Infect. Immun. **35:**270–275.
10. **Knittel, M. D.** 1975. Occurrence of *Klebsiella pneumoniae* in surface waters. Appl. Microbiol. **29:**595–597.
11. **Knittel, M. D., R. J. Seidler, C. Eby, and L. M. Cabe.** 1977. Colonization of the botanical environment by *Klebsiella* isolates of pathogenic origin. Appl. Environ. Microbiol. **34:**557–563.
12. **Matsen, J. M., J. A. Spindler, and R. O. Blosser.** 1974. Characterization of *Klebsiella* isolates from natural receiving waters and comparison with human isolates. Appl. Microbiol. **28:**672–678.
13. **Ørskov, I.** 1984. Genus *Klebsiella*, p. 461–465. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology. The Williams & Wilkins Co., Baltimore, Md.
14. **Podschun, R., A. Fischer, and U. Ullmann.** 1992. Siderophore production of *Klebsiella* species isolated from different sources. Zentbl. Bakteriol. **276:**481–486.
15. **Podschun, R., A. Fischer, and U. Ullmann.** 2000. Expression of putative virulence factors by clinical isolates of *Klebsiella planticola*. J. Med. Microbiol. **49:**115–119.
16. **Podschun, R., and H. Sahly.** 1991. Hemagglutinins of *Klebsiella pneumoniae* and *K. oxytoca* isolated from different sources. Zentbl. Hyg. **191:**46–52.
17. **Podschun, R., E. Teske, and U. Ullmann.** 1991. Serum resistance properties of *Klebsiella pneumoniae* and *K. oxytoca* isolated from different sources. Zentbl. Hyg. **192:**279–285.
18. **Podschun, R., and U. Ullmann.** 1998. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin. Microbiol. Rev. **11:**589–603.
19. **Seidler, R. J., M. D. Knittel, and C. Brown.** 1975. Potential pathogens in the environment: cultural reactions and nucleic acid studies on *Klebsiella pneumoniae* from clinical and environmental sources. Appl. Microbiol. **29:**819–825.
20. **Ullmann, U.** 1983. The distribution of *Klebsiella pneumoniae* serotypes from different sources and their sensitivity to cephalosporins. Infection **11:**S28–S31.
21. **Van Kregten, E., N. A. C. Westerdaal, and J. M. N. Willers.** 1984. New, simple medium for selective recovery of *Klebsiella pneumoniae* and *Klebsiella oxytoca* from human feces. J. Clin. Microbiol. **20:**936–941.